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Secretion of Endogenous Lectin By Chicken Intestinal Goblet Cells

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ABSTRACT The two lactose-binding lectins found in adult chicken intestine, chicken-lactose-lectin-I (CLL-I) and chicken-lactose-lectin-II (CLL-II), were localized within the vesicles of the mucin-secreting goblet cells by indirect immunofluorescence and immunoperoxidase staining methods. Attention was concentrated on CLL-II which is 200 times more abundant than CLL-I in adult intestine. The localization of CLL-II in secretory vesicles, combined with its demonstration on the intestinal epithelial surface by immune staining methods and by specific elution with lactose, suggested that at least a portion of the CLL-II in the vesicles was secreted by the goblet cells and then became associated with the mucosal surface. In support of this, treatment of isolated intestinal strips with a cholinergic agent, bethanechol (10^{-7} M) produced a small but significant increase in the amount of CLL-II that could be eluted from their surface with lactose. Secretion of lectin may occur in conjunction with mucin because both are localized in the secretory vesicles and CLL-I and CLL-II apparently bind to purified chicken intestinal mucin, which is a potent inhibitor of their hemagglutination activities. The mucin is six orders of magnitude more potent than lactose as a hemagglutination inhibitor of CLL-I or CLL-II on a molar basis, and three orders of magnitude more potent when expressed per mole of hexose. These results suggest that CLL-II, and perhaps CLL-I, are secreted from the goblet cells along with mucin. They may function in the organization of mucin for secretion and/or in its association with the intestinal mucosal surface.

A number of chicken tissues contain two lactose-binding lectins (3), designated chicken-lactose-lectin-I (CLL-I) and chicken-lactose-lectin-II (CLL-II). Both have been purified by affinity chromatography and isoelectric focusing which separates them from all other proteins including each other (3, 5). These lectins are distinct immunologically in that rabbit antisera raised against them show no cross reaction in a radioimmunoassay over a concentration range of more than three orders of magnitude (3).

CLL-II is fairly abundant in a number of embryonic tissues but is especially highly concentrated in adult intestine (3). CLL-I is also present in intestine but at <0.5% of the concentration of CLL-II (3). In previous studies with antibody to CLL-I we showed that it was concentrated in the secretory vesicles of the intestinal goblet cells (4). In the present report we show that CLL-II is concentrated at this site as well. The CLL-I previously detected at this site, although clearly a relatively minor constituent, does not appear to be an artifact caused by immunological cross-reaction of CLL-II with anti-serum raised against CLL-I. Both lectins interact with purified

chicken intestinal mucin which is a potent inhibitor of their hemagglutination activities. Direct studies with the more abundant lectin, CLL-II, indicate that it is secreted onto the intestinal mucosal surface, presumably along with mucin.

MATERIALS AND METHODS

Lectin Purification and Assay

Lectins were extracted from adult chicken intestine essentially as described previously (5). Intestinal tissue was excised, cut open and rinsed with cold saline. The intestinal mucosa was scraped off with a microscope slide and homogenized for 1 min in a Sorvall Omni-mixer (DuPont Instruments, Newtown, Conn.) at 4°C in 9 vol of 75 mM NaCl, 75 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ pH 7.2, containing 4 mM β -mercaptoethanol, 2 mM EDTA and 300 mM lactose. Lectins were purified by affinity chromatography on asialofetuin-derivatized Sepharose with specific elution with 300 mM lactose (5). Separation of CLL-I and CLL-II was achieved by preparative isoelectric focusing using pH 3–10 ampholytes (5).

Hemagglutination of trypsinized erythrocytes was assayed by serial twofold dilutions of lectin preparations as described (4). Hemagglutination titer is the highest dilution causing agglutination. Inhibition of hemagglutination was assayed by addition of test substances to assay wells in the place of saline in control wells. Minimal inhibitory concentration is expressed as the reciprocal (titer $^{-1}$) of

the highest dilution of a test substance causing reduction of lectin titer by one step. Hexose concentration was estimated by the anthrone method (13). Lactose, bovine submaxillary mucin, and crude porcine gastric mucin were obtained from Sigma Chemical Co. St. Louis, Mo.

Immunological Studies

Antisera to CLL-I and CLL-II and radioimmunoassays for the lectins have been described (3, 4).

Fixation, ultracryotomy, indirect immunofluorescent staining and photomicroscopy were all done as described previously (4). Indirect antibody staining by the peroxidase-anti-peroxidase method was performed by the method of Sternberger (14). All staining experiments were performed using a gamma globulin fraction prepared from the immune serum by precipitation in 33% saturated ammonium sulfate. The gamma globulin was redissolved in the original volume of the serum from which it was derived. In all experiments the gamma globulin solutions were diluted 1:40 before application to the sections. Controls for staining experiments included sections stained with preimmune gamma globulin and with adsorbed immune gamma globulin. For antibody adsorption, immune gamma globulin was mixed with purified lectin that had been boiled for 10 min, then used to stain sections at the same final dilution as unadsorbed gamma globulin. Boiling the lectin prevented binding of lectin to sugar residues in tissue sections, but maintained antigenic activity.

Purification of Intestinal Mucin

Chicken intestinal mucin was purified by a slight modification of the method of Forstner, et al (10). Chicken small intestine was excised, cut open, washed under running tap water, and the mucosa was scraped off with a microscope slide and homogenized exactly as described for lectin purification. The homogenate was centrifuged for 60 min at 100,000 g, the supernatant was applied to a column of Sepharose 4B equilibrated with 0.15 M NaCl and fractions were eluted with saline. Column fractions were dialyzed exhaustively against 0.15 M NaCl to remove lactose and other small molecules. Fractions were assayed for lectin inhibitory activity, lectin activity, OD₂₈₀, protein (6), and hexose concentration (13). Alkaline hydrolysis of O-glycosidic linkages in the purified intestinal mucin was performed by incubation in 0.1 N NaOH at 37°C for 48 h (12). Periodate oxidation of intestinal mucin was performed by incubation of mucin with 0.1 M sodium metaperiodate for 24 h at 4°C in the dark. The reaction was terminated with 0.1 M ethylene glycol and samples were dialyzed exhaustively. Controls were treated identically except for omission of sodium metaperiodate.

Lectin Elution from Intestinal Mucosal Surface

Intestine was excised, washed with saline at 4°C to remove luminal contents, and cut into 1-cm strips. Strips were incubated with 5 ml of 75 mM NaCl, 75 mM Na₂HPO₄/KH₂PO₄, pH 7.2 containing 4 mM β-mercaptoethanol, 2 mM EDTA and various additions of sugars as indicated. Incubation was carried out for 15 min at 4°C with gyration at 100 rpm. Supernatants were collected and assayed in duplicate for CLL-I and CLL-II by radioimmunoassay. Intestinal strips were weighed and, in some cases, homogenized in lactose-containing buffer for total lectin extraction and assay.

Lectin Secretion In Vitro

To assay lectin secretion in vitro, intestinal strips were placed in short term organ culture by a modification of the method of MacDermott et al (11). Intestine was dissected, washed with iced saline, cut into 1-cm strips and washed with 30 mM lactose in saline. Strips were then incubated in tissue culture medium at 37°C in a 5% CO₂ atmosphere with shaking at 100 rpm. The tissue culture medium consisted of 90% chloromycetin-free Trowel's T-8 Medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.) with lactose added to 30 mM and 10% fetal calf serum supplemented with penicillin, streptomycin, and mycostatin. After incubation for 30 min the medium was harvested and assayed for lectins by radioimmunoassay.

RESULTS

Specificity of Antisera Raised Against CLL-I and CLL-II

In the previous report we showed that CLL-I and CLL-II could be completely distinguished by the antisera we raised (3). Based on these results it would be expected that immunohistochemical staining with these antisera would be specific for

one or the other lectin. However, it remained possible that other antigens in the tissue might react with these antisera. To evaluate this we extracted lectin with buffer containing 0.3 M lactose, in the usual manner, then washed the pellet once and reextracted with 0.5% Triton X-100. The latter extract contained only about 3% of each antigen in the original extract, as measured by radioimmunoassay (3). This could be attributed to residual lectin in the pellet because the same amount was released by reextraction without detergent. Having established that virtually all the antigen is solubilized by our standard extraction procedure, we then showed that it all binds to an affinity column (Fig. 1). Therefore all the antigen in the tissue appears to be lectin.

Immunohistochemical Localization of CLL-II and CLL-I

CLL-II was localized immunohistochemically in intestinal slices using fluorescence and peroxidase techniques (Fig. 2). With both techniques the antigen was found to be localized within secretory vesicles in the intestinal goblet cells (Fig. 2a, c). Antigen was also detectable on the intestinal mucosal surface (Fig. 2a, c). Adsorption of anti CLL-II gamma globulin with inactivated CLL-II eliminated the specific antibody staining (Fig. 2b) whereas reaction with a large excess of inactivated CLL-I had no effect (not shown).

CLL-I was also found to be localized in the secretory vesicles of the intestinal goblet cells (Fig. 3a). Although the CLL-I concentration in intestine is only 0.5% that of CLL-II, staining with equivalent concentrations of gamma globulin directed against them gave similar staining intensity. This may be due in part to the much greater potency of the antiserum raised against CLL-I, as suggested by the concentrations required to precipitate equivalent amounts of antigen in the radioimmunoassay (3). It may also reflect the relative denaturation of antigenic activity by the fixation procedure and the relative loss of antigen during fixation.

The immunohistochemical reaction with anti CLL-I was blocked by reacting antilectin gamma globulin with 15 μg of pure inactivated CLL-I (Fig. 3b) but not by 15 times as much inactivated CLL-II (Fig. 3c). These adsorption experiments indicate that the staining is specific for CLL-I. It is not due to contamination of the antiserum raised against CLL-I with

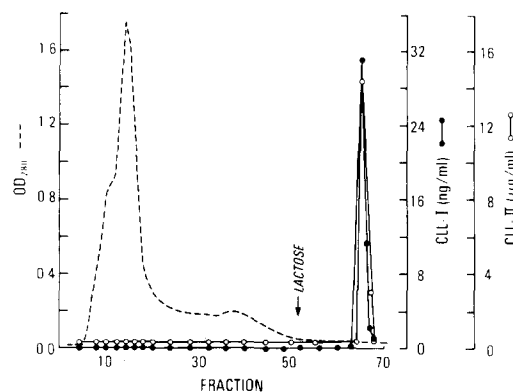


FIGURE 1 Purification of lactose-inhibitable lectins from adult chicken intestine by affinity chromatography on asialofetuin-Sepharose. Crude extract from which lactose had been removed by dialysis was applied to the column. Lactose (0.3 M) was applied to the elution buffer at the point indicated by the arrow. Recoveries were 81% and 96% for CLL-I and CLL-II respectively. No immuno-reactive material passed through the column without binding.

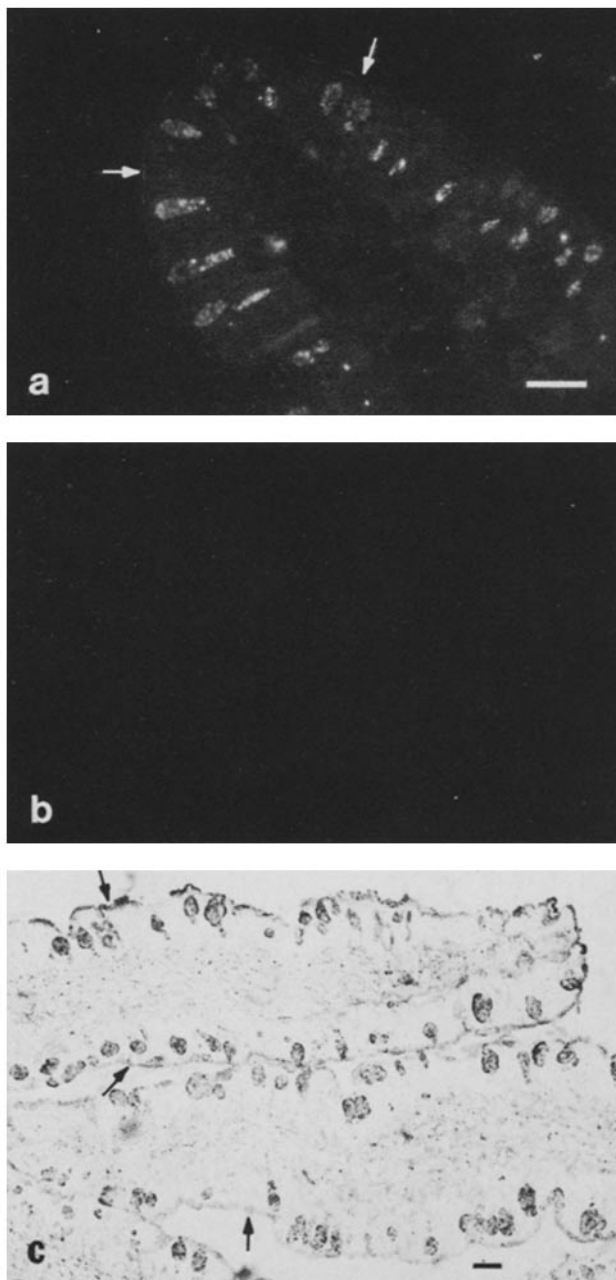


FIGURE 2 Localization of CLL-II in sections of adult chicken intestinal villi. (a) Indirect immunofluorescence staining with anti-CLL-II gamma globulin. (b) Staining with anti-CLL-II gamma globulin adsorbed with 60 μ g boiled CLL-II. (c) Indirect immunoperoxidase staining with anti-CLL-II gamma globulin. Arrows indicate staining on the luminal surface. Bars, 10 μ m.

antibodies directed against CLL-II, as already inferred from studies with the radioimmunoassays (3). Although staining of CLL-I in the vesicles was quite intense there was little or no detectable CLL-I on the intestinal mucosal surface by either the fluorescence (Fig. 3a) or the peroxidase (Fig. 3d) technique. This negative finding could reflect a true absence of CLL-I from the mucosal surface, its rapid loss *in vivo* or technical factors such as poor fixation when it is at this site.

Interaction of CLL-I and CLL-II with Chicken Intestinal Mucin

Because CLL-I and CLL-II were concentrated in vesicles of

the goblet cells, a site known to be rich in intestinal mucin, the possibility that the lectins interact with mucin was raised. To evaluate this we purified mucin from extracts of chicken intestine using a gel filtration technique similar to that developed by Forstner et al (10). In the experiment described in Fig. 4 we evaluated each fraction from the gel filtration column for hemagglutination activity and also as an inhibitor of hemagglutination activity. Whereas all the hemagglutination activity of the extracts was in the included volume, as would be expected from the low molecular weight of the lectins, all the hemagglutination inhibitory activity was in the void fractions, as would be expected from the known very high molecular weight of intestinal mucins (9). When measured by radioimmunoassay only about 2% of the CLL-II was found in the void fractions along with mucin. However, in other experiments, in which intestinal mucosa was extracted without lactose and applied to an identical column, about half the extracted CLL-II was found in the void fraction along with mucin. In Fig. 4 the high lactose concentration in the extract apparently blocked this interaction.

To evaluate the possible interactions of the purified chicken intestinal mucin with CLL-I and CLL-II we observed its effect on the hemagglutination activity of these purified lectins. The chicken intestinal mucin, like commercial mucin preparations from cow and pig, was a very potent inhibitor of the hemagglutination activities of both CLL-I and CLL-II (Table I). The inhibitory activity of the purified mucin was stable to boiling for 10 min, but was abolished by periodate oxidation, supporting the inference that the inhibitory activity is due to the carbohydrate in the mucin preparation. Whereas the chicken preparation was 4–8 times more potent than the commercial preparations based on hexose determination, this need not be taken as evidence for a species-specific interaction between chicken mucin and chicken lectin, but could rather be due to the relative crudeness of the commercial preparations. It is notable that the inhibitory activity of the chicken mucin is 1,000 times greater than that of lactose, when expressed per microgram hexose. Given the very high molecular weight of the mucin, it is $\sim 10^6$ times as potent as lactose on a molar basis.

The far greater potency of mucin compared with lactose could be due to the presence within the mucins of some saccharide residues highly specific for the binding sites of the lectin and/or to the polyvalency of the mucin molecules. Polyvalency, i.e. the large number of sugar chains per mucin molecule that might react with the lectin, could lead to simultaneous binding of lectin molecules at two sites which might markedly diminish the probability of dissociation of the lectin from the mucin molecule. In an attempt to evaluate the importance of polyvalency we degraded the mucin molecules with alkali, releasing individual saccharide chains. The efficacy of the degradation is shown from the finding that lectin inhibitory activity after hydrolysis was largely included in the gel filtration column (Fig. 5). Because alkaline hydrolysis does not break peptide bonds, these small fragments must be single sugar chains. The hydrolyzed preparation had about twice the total inhibitory titer of the unhydrolyzed preparation when expressed per microgram hexose, and assayed with CLL-II (Fig. 5). Because mucin polyvalency was markedly diminished by hydrolysis, the results suggest that the great potency of the mucin as an inhibitor is due to the substantial affinity of some of its saccharide residues for the active sites of CLL-II. In other experiments similar results were found with CLL-I. It is notable that the alkaline hydrolysis specifically degrades *O*-glycosidic

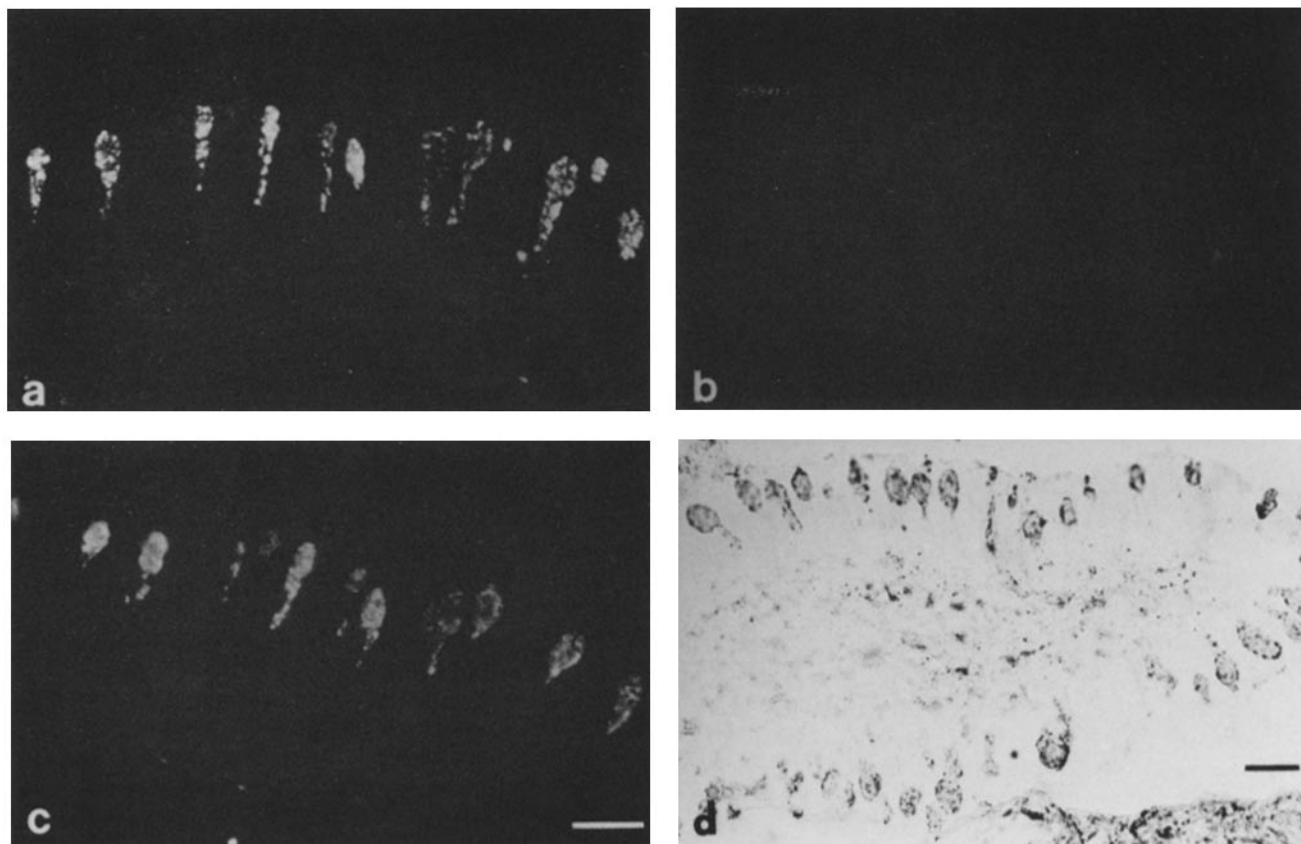


FIGURE 3 Localization of CLL-I in sections of adult chicken intestinal villi. (a) Indirect immunofluorescence staining with anti-CLL-I gamma globulin. (b) Staining with anti-CLL-I gamma globulin adsorbed with 15 µg boiled CLL-I. (c) Staining with anti-CLL-I gamma globulin adsorbed with 200 µg boiled CLL-II. (d) Indirect immunoperoxidase staining with anti-CLL-I gamma globulin. Bars, 10 µm.

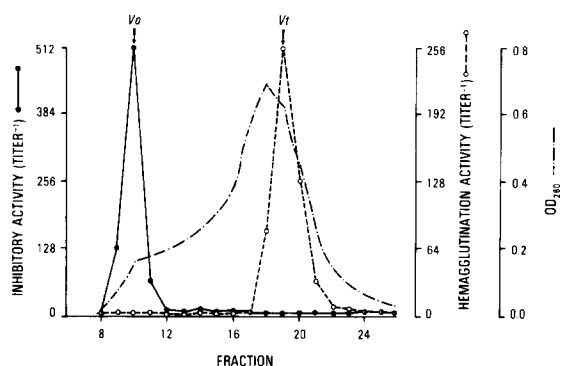


FIGURE 4 Purification of chicken intestinal mucin by gel filtration chromatography on Sepharose 4B. A lactose extract of intestinal mucosal scrapings was applied to a column of Sepharose 4B equilibrated with 0.15 M NaCl. Fractions were assayed for OD₂₈₀ (—○—), lectin inhibitory activity (●—●) and hemagglutination activity (○---○), the latter after dialysis to remove lactose. V₀, void volume. V_i, included volume.

linkages, like those known to be present in mucins (7, 12), further supporting the inference that the inhibitory activity is, indeed, due to mucin.

To evaluate the degree of possible interaction between lectin and mucin within the goblet cell vesicles we compared their amounts. 1 ml of a crude extract prepared in the usual manner contains ~30 µg of CLL-II protein and 100 µg of mucin hexose. Assuming a mol wt of 14,000 for CLL-II (5) and of 180 for a

TABLE I
Relative Potency of Lectin Inhibitors

Inhibitor	Minimal inhibitory concentration	
	CLL-I	CLL-II
	µg hexose/ml	
Lactose	120	80
Bovine submaxillary mucin	0.8	0.8
Porcine gastric mucin	0.8	0.4
Chicken intestinal mucin	0.1	0.1

Minimal concentrations of lactose and mucins that inhibited hemagglutination activity by 50% (one step) were determined by reacting serial twofold dilutions with lectin concentrations close to the agglutination end point. The minimal inhibitory concentration of chicken intestinal mucin molecules is 8×10^{-11} M, assuming a mol wt of 10^6 . When calculated in terms of mucin hexose residues, the minimal inhibitory concentration is 4×10^{-7} M. The assay wells in which 50% inhibition was determined contained 2×10^{-8} M CLL-II, assuming a mol wt of 14,000, and 5×10^{-10} M CLL-I, assuming a mol wt of 30,000.

hexose residue there are ~280 mucin hexose residues in the extract for each lectin residue. Assuming there are 10 hexose residues per saccharide chain on mucin there would be 28 sugar chains in the cell for each lectin molecule. All vesicular lectin could, therefore, be accommodated by mucin, although the amount actually bound would depend on their affinity.

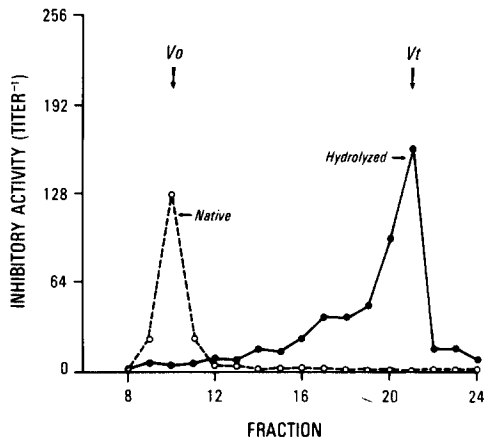


FIGURE 5 Chromatography of alkaline hydrolyzed (●—●) or untreated native (○---○) intestinal mucin on Sepharose 4B. V_0 , void volume. V_t , included volume.

Evidence for Secretion of CLL-II

Immunohistochemical studies (Fig. 2) indicated the presence of CLL-II on the intestinal mucosal surface. Given the fact that CLL-II is so concentrated in secretory vesicles and that mucin is known to be secreted into the intestinal lumen, it seemed likely that CLL-II would be secreted along with it. Were this the case one would expect that CLL-II could be eluted from the intestinal mucosal surface with lactose. As expected, low concentrations of lactose eluted considerable CLL-II from strips of intestine, whereas sucrose, a disaccharide that does not react with the active site of the lectin, did not elute it (Fig. 6). The maximal amount of CLL-II that could be eluted from the mucosal surface represented ~5% of the total intestinal content of CLL-II, as measured by the total amount of CLL-II that could be extracted by homogenizing the strips of intestine in 0.3 M lactose. Elution in this manner did not completely remove CLL-II on the intestinal mucosal surface because it remained detectable there by immunohistochemical observation with the peroxidase method.

We considered the possibility that the CLL-II eluted from intestinal strips with lactose was caused by cell breakage and release of intracellular lectin, rather than by its removal from the mucosal surface. To evaluate this we compared the specific activity of CLL-II eluted from intestinal strips with 30 mM lactose (72 μ g lectin/mg protein) with that eluted with 30 mM sucrose (8 μ g lectin/mg protein) and with the lectin specific activity when mucosal cells were homogenized in 300 mM lactose to disrupt cells and vesicles and quantitatively release lectin (20 μ g lectin/mg protein). The much higher specific activity of the lactose-eluted material suggests that a pool of protein enriched in CLL-II is actually being washed off the intestinal mucosal surface by this treatment.

In contrast with CLL-II, no detectable CLL-I was eluted from the intestinal mucosal surface. The lower limits of detection of this assay were such that if only 0.5% of the total intestinal CLL-I were on the surface we could have detected it. These results, like the immunohistochemical studies (Fig. 3) do not support the association of CLL-I with the intestinal mucosal surface. If it is secreted along with mucin and CLL-II apparently it does not remain associated with the mucosal surface.

We also directly tested secretion of CLL-II from intestinal strips by incubating them with a cholinergic secretagogue,

bethanechol, in the presence or absence of an anticholinergic compound, atropine. Low concentrations of bethanechol significantly increased the appearance of CLL-II in the incubation medium (Table II). Atropine partially reduced the bethanechol effect so that the group that had both drugs did not differ statistically from either controls or the group that received only bethanechol (Table II). Attempts to measure CLL-I in the secreted material were unsuccessful. This does not exclude secretion of CLL-I along with CLL-II and mucin, because we were working at the lower limits of sensitivity of the assay for CLL-I.

DISCUSSION

These results show that two lactose-inhibitable lectins in chicken intestine, CLL-I and CLL-II, are both localized in secretory vesicles of the goblet cells of the intestinal mucosa. Because both also react with chicken intestinal mucin, as measured by inhibition of their hemagglutination activities by this purified material, and because all three are in the vesicles at high concentration, it is likely that the lectins in the vesicles are bound to mucin. The large excess of saccharide chains in mucin compared with lectin molecules suggests that all the

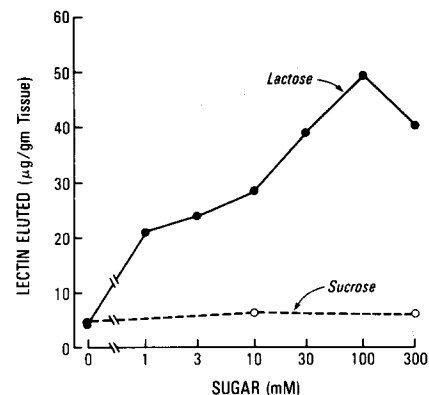


FIGURE 6 Elution of CLL-II from intestinal strips by various concentrations of lactose (●—●) or sucrose (○---○). The experiment was conducted as described in Materials and Methods.

TABLE II
Effect of Bethanechol and Atropine on CLL-II Secretion by Intestinal Strips

Addition		CLL-II Secreted %
Bethanechol	Atropine	
0	0	100 ± 11
10 ⁻⁷ M	0	160 ± 38
10 ⁻⁷ M	10 ⁻⁵ M	121 ± 15

Intestinal strips were incubated for 30 min in the presence or absence of the cholinergic secretagogue, bethanechol, and a cholinergic antagonist, atropine, as described in Materials and Methods. Secreted CLL-II was collected from the incubation supernatant and assayed by radioimmunoassay. The data shown are the combined results of 4-6 determinations from each of two such experiments and are expressed as percent of control lectin secretion (mean ± SE). In a typical experiment, 20-40 μ g CLL-II was secreted by 1.0 g of intestinal tissue in the 30-min time period. Bethanechol significantly increased CLL-II secretion ($p < 0.05$, Mann-Whitney U test). Atropine addition apparently reduced this effect, but the samples containing both bethanechol and atropine were not significantly different from either the bethanechol treated samples or the controls.

lectin might be bound to mucin, if the affinity were high enough. Because there are fewer mucin molecules than lectin molecules, more than one lectin molecule of each or both types might be associated with a mucin molecule. Because mucin molecules have so many sugar chains, and because lectins are functionally bivalent, the two could crosslink each other into a large aggregate, and be secreted together.

Direct evidence is provided that CLL-II is associated with the intestinal mucosal surface, presumably with mucin, and that it is secreted in response to incubation of isolated intestinal strips with 10^{-7} M bethanechol. The effect of the bethanechol, although statistically significant, was not great. Furthermore, inhibition of the bethanechol effect by atropine (10^{-5} M) was not statistically significant, although treatment with both drugs gave results that did not differ significantly from controls. However, when the results of these pharmacological experiments are combined with the clear demonstration of CLL-II both in secretory vesicles and on the mucosal surface, the inference that some CLL-II is secreted with mucin seems well founded. The results are also completely consistent with the possibility that much of the CLL-II in the vesicles is not released but might be recycled in some manner. It is also notable that CLL-II and mucin are readily dissociable by lactose, so that even if secreted together, they can separate.

No direct evidence was found for the association of CLL-I with the intestinal mucosal surface or for its secretion. If CLL-I is indeed also secreted, which remains a distinct possibility, it appears to be more loosely associated with the mucin and more readily lost into the intestinal lumen.

Although lectins are known to be present in a large number of plant and animal tissues, relatively little is known about their function (2). In some cases there is evidence that lectins act at the cell surface. For example, in cellular slime molds lectins appear to play a role in cell-cell adhesion (1). In plants lectins on the surface of root hairs may mediate the binding of nitrogen-fixing symbiotic bacteria (8). The present results suggest that lectins in an animal tissue may also play an extracellular function. In this instance the lectins are apparently packaged in close association with mucins and then secreted along with them. The lectins could play a role in organizing the mucin in the secretory vesicles and on the intestinal surface and/or in crosslinking mucin molecules with glycoproteins of other types on the intestinal epithelial surface. Cross-linking

may maintain an efficient coat of intestinal mucin and retard its loss into the intestinal contents. Were similar intestinal lectins produced in man, it is possible that the intestinal symptoms of a deficiency of intestinal lactase¹ may be related to the disruption of lectin-mucin or lectin-glycoprotein associations by undegraded dietary lactose, with consequent impairment in such coating functions.

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¹ For a discussion of lactase deficiency, which produces intestinal distress in many people, see G. M. Grey in: *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. 1978 McGraw Hill, New York. 1526-1536.